REMARKS

Entry of the foregoing and reexamination and reconsideration of the subject application, as amended, pursuant to and consistent with 37 C.F.R. § 1.112, are respectfully requested in light of the remarks which follow.

As noted in the Office Action Summary, claims 1-5 are pending. Claims 1-5 are amended herein. Basis for the amendments to the claims may be found throughout the specification and claims as-filed. Applicants reserve the right to file at least one continuation or divisional application directed to any subject matter canceled by way of the present Amendment.

Sequence Requirement

Per a telephone call with the Examiner of August 29, 2005, the Examiner requested that Applicants amend the specification to recite the specific sequence of the major capsid protein L1 of the human papillomavirus. Applicants have done so by way of a sequence listing, attached hereto, presenting SEQ ID NO:1. As the sequence of this capsid protein was known in the art at the time of filing, Applicants submit that it is not new matter.

Rejections Under 35 U.S.C. § 112, second paragraph

Claims 1-5 stand rejected under 35 U.S.C. § 112, second paragraph, as purportedly indefinite.

Claim 1 stands rejected for the term "intentionally modified by substitution of specific amino acids". Claim 1 is amended herein to replace this term with "modified by site-specific substitution of amino acids" in order to clarify the claimed subject matter, as supported by the specification. The claims are also rejected for the term

"type-specific epitope". Applicants note that this term is well known and has a well-established meaning, as defined by the ability to cause production of neutralizing antibodies.

Claim 2 stand rejected for the recitation of "derived". Claim 2 is amended herein to remove this term. Claim 3 stands rejected, as the metes and bounds of the intended L1 are purportedly not defined. Claim 3 is amended herein to recite SEQ IS NO:1, which is the #7 sequence of HPV16L1, in order to clarify the L1 at issue.

Claim 4 stands rejected, as it is purportedly unclear whether the fused L1 protein is the same as the original L1. Claim 4 is amended herein to clarify the intended L1 protein.

Claim 5 stands rejected for the recitation of "comprising". Claim 5 is amended herein to recite "consisting of", and to further recite pharmaceutically acceptable carriers, adjuvants and diluents in the composition. Thus, claim 5 recites elements further to those in base claim 1.

Rejections Under 35 U.S.C. § 112, first paragraph

Claims 1-5 stand rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for specifically substituted amino acids of HPV-16 L1 capable of forming a VLP while the HPV 16 L1 has lost certain epitopes, purportedly fails to provide enablement for modified L1 for any and all HPVs. Claims 1-5 stand rejected under 35 U.S.C. § 112, first paragraph, as purportedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office asserts

that only a few regions within the HPV-16 L1 region which qualify as antibody neutralizing regions have been identified.

Applicants respectfully traverse.

The skilled artisan, using the specification, would be able to determine the relevant regions.

The present claims, as set forth herein, relate to a recombinant VLP produced by removing major type-specific epitopes of the major capsid protein L1 of human papillomavirus. A cross-reactive vaccine with a broad scope of reactivity may thus be obtained by using this modified HPV-L1 protein as a carrier of HPV derived antibody epitopes.

The inventors used designed site-specific modification of amino acids to abolish major type-specific epitopes of protein L1 of HPV16. This rational design was based on 3D structural analysis and protruding amino acids in exposed loops were selected. The amino acid substitutions created were based on phylogenetic conservation and properties of the substituted amino acids, as described in the specification. The constructs were shown to be capable of forming virus like particles, VLPs, by using electron microscopy. By characterizing the HPV16 VLPs using antibodies, the depletion of said epitopes was confirmed. The HPV16 epitope depleted VLPs were able to elicit a cytotoxic T-lymphocyte response. This was demonstrated by specific lysis of MHC I expressing EL-4 cells by splenocytes from mice vaccinated with the VLPs subcutaneously. Thus, the present specification provides sufficient support. However, by way of further explanation, Applicants provide a manuscript (not yet published) by the inventors of the present patent

application, entitled "Deletion of the major neutralizing epitope of HPV 16 virus-like particles".

In light of the above, Applicants request that the rejections under 35 U.S.C. § 112, first paragraph, be withdrawn.

Rejections Under 35 U.S.C. §102

Claims 1-5 stand rejected under 35 U.S.C. § 102(b) as purportedly anticipated by Bloch et al. (WO 97/46693). To anticipate a claimed invention under §102, a reference must teach each and every element of the claimed invention. *See Lindeman Machinenfabrik GmbH v. American Hoist and Derrick Company*, 221 USPQ 481, 485 (Fed. Cir. 1984). Applicants submit that Bloch fails to recite each element of the present invention.

Bloch discloses a non-infectious virus-like particle (VLP) that contains unmodified or modified L1 proteins of a papillomavirus and a nucleic acid combined to said L1 protein. Therefore, Bloch discloses a different genetic construct from that of the presently claimed invention. With regard to present claim 3, Bloch further fails to disclose the use of the modified amino-acid positions recited in claim 3. Further, Bloch fails to disclose site-specific amino acid substitutions in protein L1, to remove type-specific epitopes causing production of neutralizing antibodies.

Claims 1-5 stand rejected under 35 U.S.C. § 102 as purportedly anticipated by Gissmann et al. (WO 96/11272). Gissman discloses recombinant papilloma virus L1 protein having deletions of one or more amino acid residues in order to produce fusion proteins capable of forming VLPs. The present claims are not directed to deletions of amino acids. Gissmann fails to recite each element of the present

invention, as Gissman fails to disclose the identification and/or production of VLPs having modified type-specific epitopes.

Claims 1-5 stand rejected under 35 U.S.C. § 102(a) as purportedly anticipated by Burger et al. (WO 99/48518). Burger discloses fusion protein consisting of at least papilloma virus L1 protein and at least one E-protein. The present invention provides the removal of papilloma virus specific epitopes by amino acid substitutions. The substitutions are based on analysis of the three-dimensional structure of the KPV16 L1 major capsid protein in order to identify putative antigenic structures and amino acids in said structures. Thus, the modification of the genetic construct as claimed, to produce VLPs according the present invention, is different from the disclosure of Burger.

Claim 1 stands rejected under 35 U.S.C. § 102(a) as purportedly anticipated by Gissmann et al. (U.S. Patent No. 6,066,324). Gissman discloses virus-like particles of papilloma. In contrast, the present invention is directed to virus-like particles of papilloma L1 protein in which amino acids are substituted in a designed manner. Thus, Gissman does not recite every element of the present invention.

Claims 1-5 stand rejected under 35 U.S.C. § 102(e) as purported anticipated by Bloch et al. (U.S. Patent No. 6,420,160). Bloch discloses virus-like particles consisting of at least a portion of L1 and a nucleic acid which is different from the genome of a papilloma virus. The modification of the L1 protein is in Bloch concerns deletions. Thus, the genetic construct of Bloch is different to that of the present invention. Further, Bloch fails to disclose how to remove major type-specific epitopes of a HPV papilloma virus by designed amino acid substitutions, as with the present invention.

Claim 1 stands rejected under 35 U.S.C. § 102(e) as purportedly anticipated by Gissmann et al. (U.S. Patent No. 6,3671,778). Gissman discloses truncated papilloma virus L1 protein, where one or more amino acid are deleted from the carboxy terminal end. Gissmann fails to disclose the virus-like particles of HPV16 L1 protein, where major type-specific epitopes are modified, of the present invention.

Claim 1 stands rejected under 35 U.S.C. § 102(a) as purportedly anticipated by Gissmann et al. (U.S. Patent No. 6,066,324). Gissman discloses papilloma virus L1 protein having deletions of one or more amino acid residues. The present invention relates to designed substitutions of amino acids in protein L1, and the present invention does not have deletions of amino acids. Thus, the product of the present invention is different from that of Gissmann. Furthermore, Gissmann fails to disclose the use of specific amino acid substitutions.

In light of the above, Applicants request that the rejections under 35 U.S.C. §102 be withdrawn.

Rejections Under 35 U.S.C. §103

Claims 1-5 stand rejected under 35 U.S.C. § 103(a) as purportedly unpatentable over White et al. (*J. of Virology*, June 1999, pages 4882-4889) and McCarthy et al. (WO 99/13056). The Office states that it would have been obvious to take the teaching of McCarthy and modify the VLPs by removing its epitopes as taught by White to decrease antibody response to the L1 and wherein the VLP can be utilized in gene therapy wherein heterologous moieties inside the VLPs can be utilized in treating diseases. Applicants traverse. To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally

available to one of ordinary skill in the art, to combine the reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. MPEP § 2143.

White discloses characterization of epitopes of human papilloma virus by using three different monoclonal antibodies. The amino acids of the HPV 16 L1 protein critical for binding to the monoclonal antibodies are identified. McCarthy discloses methods to disassembly and reassembly papillomavirus virus-like particles, VLPs. These methods are based on addition of different agents which cause correct folding of the L1 protein capable of containing moieties such as DNA or peptide.

These two references, taken in combination, fail to disclose the present invention. The amino acids disclosed by White are not those of the present invention, and neither reference provides teaching or motivation as to how to identify major type-specific epitopes as in the present invention. To this end, the present invention is directed to site-specific modification of amino acids in protein L1. These modifications abolish major type specific epitopes. A cross-reactive papilloma vaccine may then be produced by using said modified EPV-L1 protein as a carrier of surface exposed HPV derived antibody epitopes. This is not disclosed or suggested by the combined disclosures of White and McCarthy. Thus, there is no expectation of success in arriving at the results of the present invention, upon combining these references.

Claims 1-5 stand rejected under 35 U.S.C. § 103(a) as purportedly unpatentable over Christensen et al. (*J. of General Virology*, 1994, Vol. 75, pages

2271-2276), and Touze et al. (*Nucleic Acid Research*, 1998, Vol. 26, No. 5, pages 1317-1323). The Office asserts that it would have been obvious to take the teaching of Touze and modify the VLPs by removing its epitopes as taught by Christensen. Applicants traverse.

Christensen discloses neutralizing epitopes of human papillomavirus L1 protein, but fails to provide disclosure as to the position or identity of the neutralizing epitopes in protein L1. Touze does not remedy the deficiencies of Christensen. Touze discloses that papilloma like virus particles composed of L1 protein may be used as vehicles for foreign genetic material into cells. The two references, taken in combination, do not disclose or suggest modifying major type-specific epitopes in order to achieve a vehicle which, when carrying a surface exposed HPV epitope, would produce broadly cross-reactive vaccine. Because the identity and position of amino acids constituting epitopes are not disclosed in the references, the references do not even suggest or provide motivation to attempt carrying exposed HPV epitopes.

Claims 1-5 stand rejected under 35 U.S.C. § 103(a) as purportedly unpatentable over Christensen et al. (*J. of General Virology*, 1994, Vol. 75, pages 2271-2276), and McCarthy et al. (WO 99/13056). The Office asserts that it would have been obvious to take the teaching of McCarthy et al. and modify the VLPs by removing its epitopes. Applicants traverse.

Christensen discloses neutralizing epitopes of human papillomavirus L1 protein, and fails to recite or suggest the position or identity of the neutralizing epitopes in protein L1. McCarthy discloses methods for disassembly arid reassembly of virus like particles by additions of certain agents which then enable

the VLPs as carrier of foreign genetic material. The present invention is directed to the identity and position of amino acids constituting major type specific epitopes and removal of said epitope. The achieving of a vehicle which, when carrying antibody or T-cell epitopes, would produce a cross-reactive HPV vaccine without obstruction from type-specific antibodies towards the carrier itself, is unexpected. Thus, not only do the cited references fail to recite the elements of the present invention, they fail to provide an expectation of success upon modification of the references.

Claims 1-5 stand rejected under 35 U.S.C. § 103(a) as purportedly unpatentable over Carter et al. (*J. of Virology*, Nov. 2003, Vol. 77, No. 21, pages 11625-11632), and Touze et al. (*Nucleic Acid Research*, 1998, Vol. 26, No. 5, pages 1317-1323). The Office asserts that it would have been obvious to take the teaching of Touze et al. and modify the VLPs by removing its epitopes as taught by Carter. Applicants traverse.

Carter discloses L1 protein amino acid residues important in binding to monoclonal antibodies. Touze discloses papilloma like virus particles composed of L1 protein may be used as vehicles for foreign genetic material into cells. Carter and Touze fail to disclose amino acid residues identical to those of the present invention. As a result, the cited references fail to provide expectation of success, as they do not provide guidance as to how major type-specific epitopes would be removed oras to which amino acid substitutions to make in order to retain formation of virus-like particles. These references fail to disclose the property of obtaining a broadly cross-reactive vaccine, which having said modified protein L1, and carrying antibody or T-cell epitopes, will not cause obstruction from type-specific neutralizing antibodies towards the carrier itself.

In light of the above, Applicants request that the rejections under 35 U.S.C. § 103 be withdrawn.

CONCLUSION

It is respectfully submitted that all rejections have been overcome by the above amendments. Thus, a Notice of Allowance is respectfully requested.

In the event that there are any questions relating to this amendment or the application in general, it would be appreciated if the Examiner would contact the undersigned attorney by telephone at (703) 836-6620 so that prosecution of the application may be expedited.

Respectfully submitted,

BUCHANAN INGERSOLL PC
(INCLUDING ATTORNEYS FROM BURNS DOANE SWECKER & MATHIS)

Date: November 23, 2005

By: _____
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Enclosure: Exhibit A

Deletion of the major neutralizing epitope of HPV16 virus-like particles

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Abstract

The Human Papillomavirus type 16 (HPV16) is a major cause of human cancer. Effective prophylactic vaccines are based on type-specific neutralising antibodies. A major neutralising epitope is defined by the monoclonal antibody V5. To investigate the importance of this epitope for overall immunogenicity of HPV16, we engineered HPV16 virus-like particles devoid of the V5 epitope by site-directed mutagenesis of 10 non-conserved, surface-exposed residues. Removal of the V5-defined epitope had only marginal effect on antigenic reactivity with antibodies in sera from infected subjects, but affected immunogenicity in experimental immunization of mice, with reduced induction of both antibody responses and CTL-responses.

Keywords: human papillomavirus, virus-like particles, V5

1. Introduction

The human papillomaviruses (HPVs) cause a wide variety of benign- and pre-malignant epithelial tumors. Of the almost 100 different types of HPVs that have been characterized, approximately two dozen specifically infect genital and oral mucosa [1]. The HPVs are the most common of the sexually transmitted diseases. A small part of genital infections with high-risk HPV types will progress to invasive cervical cancer [1, 2]. The most common oncogenic HPVs found in invasive cervical cancers are HPV-16, -18, -31 and -45 [3]. HPV-16 is found in about 50 % of cervical cancers, HPV-18 in about 20 % and these four types together account for over 80 % of all cervical cancers [3]. HPV has in recent years also been established as a cause of cancers of the penis, vulva, anus, vagina and oropharynx [4]. Carcinoma of the cervix is one of the most prevalent carcinoma in women worldwide and is associated with HPV in at least 90 % of the cases [5]. HPV vaccine development is therefore a prime priority of preventive cancer research today.

Comment [p1]: ref

The HPV capsids consist of 72 capsomers each containing 5 copies of the HPV major capsid protein L1 [6]. The capsid proteins will, when expressed, self assemble to form virus like particles (VLPs) i.e. particles morphologically indistinguishable from the authentic virions but lacking the potentially oncogenic viral genome [7]. These VLPs have elicited high titres of systemic neutralising antibodies in several animal model systems (rabbits, cows, rhesus monkeys) even when injected in the absence of an adjuvant [8]. The neutralising antibodies primarily recognise conformational L1 epitopes displayed on intact VLPs or virions [6]. It has previously been shown that most antibodies in human sera that are reactive with intact HPV16 capsids could be blocked by a single monoclonal antibody V5 [9]. Vaccination with VLPs has been shown to be highly efficient for protection against subsequent challenge with both cutaneous- and mucosal papillomaviruses as well as for protection against natural HPV infection in human trials [10, 11]. The protection and the neutralising antibodies are only effective for the specific virus type immunised with, owing to the presence of a strongly immunodominant type specific epitope [9, 12-14]. Apart from the fact that HPV VLPs are highly efficient in eliciting a high-titer neutralizing antibody response, VLPs are also highly efficient in eliciting a cytotoxic T lymphocyte (CTL) response [15-18] and VLP-based vaccines have been found to be highly efficacious in preventing and treating transplantable cancers in several mouse models, in spite of the fact that immunisation is made with an exogenous protein [15, 16]. The high antibody immunogenicity appears to be due to, at least

in part the presence of closely spaced repetitive units of the antigen [19] and the CTL immunogenicity appears to be due to the preservation of an active mechanism for infection of the cell (designated pseudo-infection, as no viral genome is introduced) which results in that the capsid protein is processed and presented in the MHC class I presentation pathway [18, 20, 21]. VLPs can therefore be used as a vehicle for efficient immunogenic delivery of any antigen [13, 18]. Efficient immunisation using HPV VLPs carrying foreign antigens has been demonstrated in several systems, e.g. melanoma antigens and human immunodeficiency virus antigens [22-24].

Pre-existing neutralizing antibodies against HPV VLPs severely interferes with the induction of CTL-responses, as originally found using E7-containing HPV chimeric VLPs in a murine model system [25]. A possible solution to this problem would be to remove the neutralising epitopes from the VLPs. Therefore, our primary aim was to obtain HPV16 L1 VLPs devoid of the major neutralising epitope. Such VLPs might be able to evade a neutralising antibody response, but would be predicted to still be able to deliver defined CTL-epitopes to the immune system. Our more general aim was to provide more detailed knowledge of the structure of the immunodominant type-specific neutralizing epitope defined by V5, an issue of fundamental interest for HPV vaccinology.

2. Materials and Methods

2.1 Software for visualisation of HPV16 L1 protein and space model building

The programs used for visualizing the L1-protein structure were Wisconsin GCG, Swiss PDB-viewer, Rasmol, WhatIF, Antigenic and Protscale. The atomic coordinates of a L1-monomer from Chen *et al* [6] were used.

2.2 Selection of residues for mutagenesis

Probable surface exposure of residues was evaluated through visual studies of the L1-pentamer in Rasmol and Swiss PDB-viewer. Antigenicity predictions were done using Wisconsin GCG, ProtScale and WhatIF software. The variability of residues between HPV types was determined by comparing L1 sequences of different HPV-types using the 1997 human papillomavirus sequence database (http://hpv-web.lanl.gov).

2.3 Construction of V5-epitope depleted HPV16 VLPs

A truncated chemically synthesized HPV16 L1 gene lacking the C-terminal 34 residues was purchased from Interactiva Biotechnologie GmbH, Ulm, Germany. The gene was codon-adapted for expression in *E.Coli*. Truncations of up to 30 C-terminal residues had been reported to have little or no effect on the stability or solubility of the expressed protein, whereas C-terminal deletions longer than 30 residues had been reported to render the protein extremely sensitive to proteases and unstable [6]. Therefore, an additional 14 amino acids segment codon-adapted for expression in *E.Coli* was ligated to the construct, creating an HPV16 L1 gene lacking only 20 residues in the C-terminus..

All mutations in the truncated HPV16 L1 sequence were performed using Stratagene Quickchange Multi Site-directed Mutagenesis Kit (Cat No. 200514). All constructs were sequenced to confirm mutations. Constructs were then cloned into the *BamHI/NotI* sites in the expression vector pVL1393 (Pharmingen, Cat No. 21486P) using standard molecular biological cloning techniques.

2.4 Generation of a truncated ovalbumin-L1 HPV16 constructs

Oligonucleotides coding for the ovalbumin CTL-epitope (SIINFEKL) and the helper-epitope (TEWTSSNVMEERK) were purchased from Shafer-N, Copenhagen Denmark. The peptides fit the binding motifs for the murine MHC-I molecules K^b and D^b. The oligos were cloned

into the C-terminus (nucleotide 1445-1558) of the truncated –20 amino acid HPV16 L1 major capsid protein using standard molecular biological techniques.

2.5 Generation of Recombinant Baculoviruses

Spodoptera frugiperda (Sf9) cells were grown in culture at 27°C Sf-900II medium (Life Technologies. Cat No. 10902-096) supplemented with 4% FCS and 2 mM glutamine. Five micrograms of baculovirus transfer plasmid (pVL1393 Cat.No. 21486P, Pharmingen) containing truncated- wild type HPV16 L1, V5-epitope depleted HPV16 L1, wild type HPV16 L1 ovalbumin or V5-epitope depleted HPV16 L1 ovalbumin. was used to transfect Sf9 cells together with 0.5 µg of linearized Baculo-Gold DNA (Pharmingen, Cat No. 21001K). The pVL1393 baculovirus transfer vector contains the complete polyhedrin gene locus of the *Autographa californica* nuclear polyhedrosis virus cloned into the pUC8 vector, but lacks the polyhedrin gene coding region. After plaque purification of recombinant clones, high-titer recombinant virus was generated. Cultures of High Five insect cells were infected with recombinant high titer baculovirus at a multiplicity of infection of 10. After 72 h at 27°C, cells were harvested [26-28].

2.6 Purification of HPV VLPs

Infected insect cells were harvested, pelleted, and resuspended in 10 ml of phosphate-buffered saline (PBS) pH: 6.0 containing 0.5% Nonidet P-40. After a 30-minute incubation on ice the cells were sonicated for 4x20 seconds at 60% maximal power (Virsonic 300, Virtis Company.Inc). Lysates were loaded onto a 40% (wt/vol) sucrose cushion and centrifuged in a Beckman swinging-bucket SW28-rotor for 3 h at 125 000 x g at 4°C. Pellets were resuspended in 3 ml of PBS containing 32% CsCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES and sonicated for 3 s at 60% maximal power. The suspension was then placed in 13.4 ml Beckman Quickseal tubes. Samples were centrifuged in a Beckman swinging-bucket SW28-rotor for 20 h at 150 000 x g at 4°C. Gradients were fractionated by puncturing tubes on top and bottom with a 21-gauge needle, and 10 drops of each fraction was analyzed. The densities were determined by refractometry. Positive fractions were dialyzed against PBS containing 0.5M NaCl, 5 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$.

2.7 Western blot analysis

Reduced VLP-samples were run on NuPage 4-12% Bis-Tris gels (Invitrogen Cat No. NP0321BOX) and blotted to nitrocellulose membrane (Invitrogen, LC2001). The membranes

were blocked overnight with 5% nonfat dry milk and incubated with anti-HPV-16 L1 monospecific antipeptide antibodies (L1-16A or L1-30A) [29]. Following incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnologies, Cat No. Sc2004), the membranes were developed with the ECL-Plus system (Amersham Biosciences, RPN2132).

As positive control, purified baculovirus-expressed VLPs of HPV16 (obtained from Dr. John T. Schiller, Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, USA) were used.

2.8 Mouse immunization with VLPs

Eight to twelve-week-old female C57BL/6 mice (Taconic M&B) were injected subcutaneously with 10 µg of truncated wild type-, V5-epitope depleted-, ovalbumin- or ovalbumin-V5-epitope depleted HPV 16 VLPs administered with an equal volume of Complete Freunds Adjuvant (CFA, Lot No. 2337761). A group of control mice was injected with PBS-CFA only. Each group consisted of 5 mice. The mice were boosted day 14 and 28 with VLPs in solution with Incomplete Freunds Adjuvant (Lot No. 67144685). The mice were bled through vena saphena on day 27, 35 and 42.

2.9 ELISA

ELISA plates were coated overnight at 4°C with baculovirus-produced VLPs (V5-epitope depleted or wild type) in cold PBS pH 7.2. As negative control, disrupted VLPs generated through incubation at room temperature for 4 h in carbonate buffer pH 9.6 were used. After washing of the plates four times with PBS-0.5% Tween 20 (PBS-T), they were blocked with PBS supplemented with 10% horse serum (10% HS-PBS) at room temperature for 1 h. The V5 monoclonal antibody or D9 monoclonal antibody (kindly provided by Dr. Neil Christensen, Departments of Pathology, Microbiology and Immunology, Pennsylvania State University College of Medicine, Hershey, USA) diluted in 10% HS-PBS were incubated for 1 h at room temperature. Sheep anti-mouse-IgG horseradish peroxidase conjugate (Amersham, NP9310) 50 µl (diluted 1:2.000 in 10% HS-PBS) was added to the ELISA plates after washing four times with PBS-T. The plates were incubated for an hour at room temperature and washed four times with PBS-T. 3,3',5,5'-tetramethyl-benzidine substrate (100 µl per well) (TMB, Pharmingen, Cat No. 555214) was added and was allowed to react for 15 min. The reaction was stopped by the addition of 100 µl of 1 M HCl/well, and the optical density was read at 450 nm. In case of ELISAs with sera from immunized mice, serum were added to

wells at dilutions from 1:100 – 1:218 700 (diluted in 10% HS-PBS) and incubated for 1 h. After washing bound antibody was detected using sheep anti-mouse-IgG horseradish peroxidase conjugate (Amersham Biosciences, NP9310, diluted 1:2,000 in 10% HS-PBS). In case of ELISAs with human serum, the human sera were incubated for 2 h at room temperature. After washing a mouse monoclonal anti-human IgG (gamma-chain-specific) antibody (Eurodiagnostica, diluted 1/800 in 10% HS-PBS) was added and allowed to react for 90 min at room temperature. As secondary antibody anti-mouse Ig G-HRP was used (Amersham Biosciences, NP9310, diluted 1:2,000 in 10% HS-PBS). When heparin coated ELISA plates [30] were used, the plates were incubated with VLPs for 1 hour at 37°C. Blocking proceeded for 15 minutes at 37°C and samples were incubated for 1 hr at 37°C.

2.10 Cytotoxic T-Lymphocyte Assay

Eight to twelve-week-old female C57BL/6 mice (Taconic M&B) were injected subcutaneously with 10 µg of wild type-, V5-epitope depleted-, ovalbumin- or ovalbumin-V5-epitope depleted HPV 16 VLPs administered with an equal volume of Incomplete Freunds Adjuvant (IFA, Lot No. 67144685). One group of control mice was injected with PBS-IFA only and one group with 100 µg ovalbumine H2-K^b-restricted CTL-epitope (SIINFEKL) and Th-peptide (TEWTSSNVMEERK) (Shafer-N, Copenhagen Denmark). Each group consisted of 4 mice. The mice were boosted day 14 with VLPs in solution with IFA. Three weeks after booster immunization mice were sacrificed by cervical dislocation and spleen cells were isolated. After incubation in nylon wool columns for 1 h at 37°C and 5% CO_2, enriched T-cells were washed through the column with complete cell culture medium (RPMI 1640 medium, including 10% FCS, 1 mM Na-Pyruvate, 5 µM Mercaptoethanol, 10 mM HEPES and 0.1 mg/ml gentamicine). Cells were cultured for 5 days at 37°C and 5% CO_2 in RPMI 1640 medium, including 10% FCS, 1 mM Na-Pyruvate, 5 µM Mercaptoethanol, 10 mM HEPES and 0.1 mg/ml gentamicine. IL-2 (Chiron, E100F28/4) was added to wells on day 3. On day 5 erythrocytes, dead cells and debris was removed through centrifugation with Lympholyte-M (Cat. No. CL5030, Cedarlane Laboratories). Specific cytolytic activity was determined by a ^{51}Cr release assay. Target mouse ascites lymphoma lymphoblast EL-4 cells (H-2b) were labelled with ^{51}Cr (100 µCu) for 1 h at 37°C and washed three times. Half of the EL-4 cells were further loaded with ovalbumin peptides. Target cells (2500 cells per well) were then incubated with effector cells at different effector/target ratios in V-bottomed 96-well microtiter plates for 6 h at 37°C. Supernatant was collected and ^{51}Cr release was

quantified using a γ counter. Specific lysis was calculated using the formula [(experimental release – spontaneous release)/(maximum release – spontaneous release)] x100. Spontaneous release was determined in control microcultures containing ^{51}Cr-labeled target cells in culture medium with no effector cells. Maximum release was determined by lysing ^{51}Cr-labeled target cells with 0.5% NP-40.

3. Results

3.1 Identification of residues targeted for mutagenesis

The C-terminal truncated ~20 amino acid HPV16 L1 major capsid protein (Ac: af393502.1) (Protein Data Bank entry 1DZL) was analysed using 3D structural analysis for identification of putative antigenic structures.

The main criteria for selection of residues for mutation were: i) Amino acids were to be surface exposed in the 3D-models, as illustrated by figure 1 and table 1B. In addition surface probability was estimated using hydrophilicity/hydrophobicity plots (Hopp-Woods) (table 1B). ii) Amino acids were to be variable among immunologically distinct but phylogenetically closely related HPV-types. The selected amino acids were found to be variable between closely related HPV-types (table 1A). iii) Amino acids were to be directed outwards and have little or no interaction with neighbouring amino acids (exemplified in Figure 1). iv) Mutations of selected amino acids should have no effect on the 3D-structure of the HPV-pentamer. The mutations were simulated using Swiss PDB-viewer: none of the mutations seemed to affect the 3D-structure.

We chose to substitute larger amino acids for smaller and expected an effect due to chemical change. The amino acids GLY, SER and ALA are uncharged and relatively small amino acids and predicted to be less antigenic. ALA is weakly hydrophobic and smaller than any other amino acid except GLY. ALA substitution is not expected to have major effects on secondary structure, but will remove any chemically significant side chain interactions. Mutation to GLY was only performed in regions predicted to have no secondary structures. At position 285, different HPV-types contained different residues but hydrophilicity was maintained. Therefore, we opted for SER substitution at this residue. At position 348 many amino acids were hydrophobic and we therefore chose ALA substitution. Thus, the following amino acid substitutions were made: aa56 ASN→GLY, aa138 ASP→GLY, aa270 ASN→GLY, aa 285 ASN →SER, aa348 ILE→ALA, aa349 SER→GLY, aa 350 THR→GLY, aa351 SER→GLY, aa352 GLU→GLY and aa 353 THR→GLY (table 1).

3.2 Characterisation of purified VLPs

VLPs were isolated from insect cells and purified. The fractions were analysed in western blot using the anti-HPV16 L1 antipeptide antibody L1-16A or L1-30A [29]. The wild type- and V5-epitope depleted truncated HPV16 L1 showed bands comigrating with the HPV16 L1

control (Figure 2). The wild type- and V5-epitope depleted truncated HPV16 L1 with ovalbumin was slightly larger. Some lower molecular weight bands, presumably degradation products, were also seen (fig 2).

L1-positive fractions were absorbed onto carbon-coated grids, stained with uranyl formate and examined by transmission electron microscopy (data not shown). The VLPs exhibited an average diameter of 50-60 nm and were present as defined particles with little or no aggregation. Smaller particles and partially assembled structures were also seen.

3.3 Verification of V5-epitope depletion and assembly by ELISA

To verify the assembly of wild type particles and the V5-epitope depletion of mutant particles, the monoclonal antibodies V5 (against major neutralising epitope, reacts with intact VLPs of all HPV16 strains), E70 (another HPV16 neutralizing monoclonal antibody that reacts only with some strains of HPV16) [31] and D9 (binds disrupted HPV16 VLPs) were reacted with the different VLPs. The truncated wild type HPV16 L1 VLPs had high reactivity to V5 (Table 2). The truncated wild type HPV16 L1 VLPs with ovalbumin did not react as strongly. None of the V5-epitope depleted VLPs bound V5 (Table 2). Disrupted VLPs were used as controls and reacted with the D9 monoclonal antibodies in a comparable manner. Both truncated wild type HPV16 L1 VLPs and the V5-epitope depleted HPV16 bound strongly to E70.

3.4 Immunisation of C57BL/6 mice with truncated wild type- and V5-epitope depleted HPV16 L1 VLPs with and without ovalbumin and analysis of immune sera.

Four groups of C57BL/6 mice were immunised subcutaneously with 10μg of the different VLPs in PBS-complete Freunds adjuvant (PBS-CFA). Control mice were injected with PBS-CFA only. Each group consisted of 5 mice. The mice were boosted day 14 and 28 with VLPs in Incomplete Freunds Adjuvant. Antibody levels were measured by ELISA (figure 3). Antibody titers against wild-type VLPs from mice immunized with V5-epitope depleted truncated VLPs were more than a logarithm lower than from mice immunized wild type truncated VLPs.

3.5 Interaction of VLPs with heparin coated ELISA plates

To explore the ability of truncated wild type HPV16 L1 constructs to interact with heparin, we used ELISA plates coated with bovine serum albumin (BSA)-conjugated heparin. The VLPs that lacked the last 20 amino acids at the C-terminus had a weaker binding to heparin than the control "full length" HPV16 L1 VLPs did (Figure 4).

3.6 Analysis of human HPV16 serum reactivity with truncated wild type- and ITSE-depleted HPV16 L1 VLPs.

A panel of human serum samples from 49 women testing positive for HPV16 DNA in PCR of cervical samples (positive controls) and 11 women reporting no sexual experience (negative controls) [32] were tested for reactivity with control full length HPV16 VLPs and truncated wild type- and V5-epitope depleted HPV16 VLPs. All VLPs were specifically reactive with the positive control panel (Table 3a). Comparison with HPV16 neutralisation data from a previous study (30, 33]) found that the antibody reactivities increased with increasing neutralisation titers (Table 3b). The reactivity with the V5-depleted VLP was only marginally reduced (Table 3). The serologic reactivity of the truncated, V5-epitope depleted VLPs correlated well with the reactivity with similar VLPs containing V5 binding (r=0.95). The serologic reactivity to full length HPV16 VLPs also correlated well with the reactivity to truncated HPV16 VLPs (r = 0.94).The correlation between serologic reactivity to full length HPV16 L1 VLPs and truncated V5-epitope depleted HPV16 L1 VLPs was r = 0.86.

3.7 Test of V5-epitope depleted VLPs ability to induce an efficient CTL-response

To test weather V5-epitope depleted VLPs effectively elicit a cytotoxic T –lymphocyte response, C57BL/6 mice were immunized subcutaneously with the different VLPs or with PBS-IFA only (negative control) or with ovalbumin CTL-epitope and Th-peptide (positive control). Three weeks after the booster immunization mice and subsequent culturing of the spleen cells, the cytolytic activity against syngeneic EL-4 cells loaded with the ovalbumin CTL epitope peptide was analysed in a chromium release assay (Figure 5). The CTL reactivity of the mice immunized with the wild-type VLPs containing the ovalbumin epitope was rather low and mice immunized with the corresponding V5-depleted VLPs had an even lower cytolytic response (Figure 5).

4. Discussion

Knowledge of antigenic domains in papillomaviruses is important for the design of prophylactic vaccines and may be important for quality control of different batches and preparation of VLPs to be used for vaccination. New insights may also further the use of VLPs as immunotherapeutic vectors. E.g., it may enable design of VLPs that are not neutralised by neutralising antibodies to the immunodominant type-specific epitope (naturally occurring or induced by prior immunization).

We successfully designed a strategy for removal of the major type-specific conformational neutralising epitope of HPV16 (as defined by the V5 antibody). Substitution of 10 surface-exposed non-conserved residues from the 4 major surface-exposed loops resulted in VLPs with barely detectable reactivity with the V5 monoclonal antibody.

The outer surface of each L1 pentamer has five broad pockets, created by the BC-, EF-, and FG-loops. These are all putative receptor pockets [6]. Regions within the FG- and HI-loop have already been proposed as receptor sites for V5 as evidenced by the fact that hybrid VLPs that have transplanted the FG- and HI-loops into the HPV11 L1 protein have gained significant binding of the V5 antibody [12]. The V5 antibody also binds to hybrid VLPs containing only the FG loop, but it binds stronger to hybrid VLPs who contain both the FG- and the HI-loop [12], suggesting that the FG-loop contains the predominant epitope recognized by V5 but that it requires the HI-loop to maintain conformational stability. Our constructs that did not bind V5 contained only 2 mutations within the FG loop (residue 270 and 285), suggesting that these 2 residues are important for V5 binding.

We found that a truncated form of HPV16 lacking the last 20 amino acids in the C-terminus (with or without the V5 epitope) self-assembled into VLPs of similar appearance to full length VLPs in electron microscopy. Constructs containing an insert with the ovalbumin epitope formed VLPs of correct size, but also smaller, more irregular, VLPs. Thus, neither the V5 epitope deletion or the C-terminal truncation seemed to affect the assembly of VLPs. Deletions of up to 30 C-terminal residues have previously been reported to have little effect on the stability of expressed protein [6]. However, it has also been reported that truncations of the C-terminus may affect VLP-formation and stability may depend not only on the length of the insert but also on the nature of the inserted gene [24, 34].

Glycosaminoglycans (heparin, heparan sulphate etc) are candidate primary receptors for HPV VLPs [35, 36]. Modis *et al* has described the structure of the C-terminus and suggested that

large parts of it are surface exposed and antigenic [37]. Joyce *et al* has suggested that the C-terminus 15 amino acids are responsible for the interaction with heparan sulphate and identified a conserved region within the last 15 amino acids of most HPVs that could conceivably serve as or part of a heparin-binding motif [38]. Enzymatic removal of the C-terminus of HPV11 completely abolished binding to heparin [38] but a similar experiment with HPV33 did not affect binding [36]. We found that VLPs lacking the last 20 amino residues of L1 had somewhat weaker binding to heparin. We can not distinguish whether this could be due to conformational instability issues or to that the missing amino acids are part of the heparin binding site or otherwise contribute to the natural heparin binding ability of full length VLPs. Reduced binding to the HPV receptor is likely to affect the ability to induce an effective CTL-response.

We found that antibody titres to HPV16 VLPs from mice immunized with V5-epitope depleted HPV16 VLPs were at least a logarithm lower than from mice immunized with similar VLPs with V5 binding. Therefore, we conclude that the truncated V5-epitope depleted HPV16 L1 VLPs are considerably less immunogenic than wild type truncated HPV16 L1 VLPs.

Our evaluation of the serologic reactivity of V5-depleted VLPs found a high correlation to the reactivity with similar VLPs containing V5 reactivity and the detectable ELISA absorbances were only moderately reduced for the V5-depleted VLPs. The monoclonal V5 is capable of inhibiting the reactivity of a majority of reactive human sera [9], but our current results suggest that it is possible to separate the V5-epitope from the immunodominant type-specific epitope recognized by these human sera. The monoclonal antibody E.70 is also HPV16-neutralizing. E.70 is directed to a conformational epitope with the variable amino residue at 282 being critical [31]. E.70 also inhibits the reactivity of human sera to HPV16 VLPs, but to a lesser extent than V5 does [9]. The fact that E.70 only recognizes some strains of HPV16, whereas human sera (and the V5 monoclonal) appear to react with all viral variants of HPV16 [31] implies that the human serum reactive epitope is not exactly the same as the epitope recognized by E.70. It has been suggested that E.70 and V5 have overlapping epitopes. However, the mutated L1 VLPs we produced had strongly reduced V5 binding, but the binding by E70 was not measurably affected.

We tested whether V5-epitope depleted VLPs fused to ovalbumin effectively can elicit a cytotoxic T –lymphocyte response since it is a desirable property of VLPs based vehicles to be able to generate an immunological response towards an introduced peptide, without

obstruction from type specific neutralising antibodies directed towards the carrier itself. We found that VLPs with the C-terminal 20 amino acids replaced by an ovalbumin epitope have low immunogenicity in a CTL-assay, which was further reduced for the VLPs lacking the V5-epitope. The insertion of the ovalbumin epitope appears to have affected the conformation of the VLPs, as evidences by somewhat reduced V5 binding. Also, the C-terminal truncation had reduced heparin binding ability. Conceivably, the truncation and/or the insertion of the ovalbumin epitope VLPs may have affected the ability to bind to and infect cells.

In conclusion, we have designed a strategy that can successfully remove a major type-specific neutralizing epitope from HPV16 VLPs. Our removal of the V5-defined epitope appeared to affect the ability to elicit antibodies in experimental mice immunization, but had marginal effect on antigenic reactivity with antibodies in infected subjects suggesting that the V5 epitope and the epitope recognized by human sera can be separated. These findings may have important implications for the continued design of VLP vaccines as well as for monitoring of functional antigenicity of VLP preparations and associated antibody responses.

5. Acknowledgements

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from insect cells inhibits the growth of HPV16-expressing tumor cells in mice. Virology 2001;279(1):354-60.
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7. Tables

Table 1a: Shows the variability of chosen amino acids between HPV-types.

HPV16[a]	HPV33[a]	HPV35[a]	HPV58[a]	HPV31[a]	HPV52[a]	HPV6b[b]	HPV11[b]	HPV18[c]	HPV45[c]	HPV
Residue 56 (Moderately variable, surface exposed)										
N	N	D	N	N	N	-	-	G	A	
Reside 138 (Variable, surface exposed)										
N	A	T	A	S	P	-	N	N	V	
Residue 270 (Variable, surface exposed)										
N	A	T	A	S	P	P	P	T	T	
Residue 285 (Variable, surface exposed)										
N	S	T	T	T	T	S	S	P	T	
Residue 348-353 (Variable, surface exposed)										
ISTSET	TLCTQV	VSSSDS	TLCTEV	IANSDS	TLCAEV	VTTSST	VSKSAT	TQSPVP	TQNPVP	TAI
Example of Refused Amino Acids: Residue 360 (Conserved)										
F	F	F	F	F	F	Y	Y	F	F	
Example of Refused Amino Acids: Residue 361 (Conserved, proximity to residue 283, projecting towards surface)										
K	K	K	K	K	K	K	K	K	K	

[a] Phylogenic Group A

[b] Phylogenic Group B

[c] Phylogenic Group C

[d] Phylogenic Group D

Table 1b: Shows the surface probability, antigenic index and hydrophilicity of the amino acids chosen to be mutated.

Amino Acid Position	56	138	270	285	348	349	350	351	352	353
Amino Acid	N	N	N	N	I	S	T	S	E	T
Surface Prob.*	5.90	0.28	1.21	0.60	0.43	0.73	1.04	2.14	2.51	3.47
Antigenic Index**	1.70	-0.60	0.90	-0.60	-0.60	0.45	0.80	0.80	0.40	1.30
Hydro-philicity***	1.10	-0.67	0.78	-0.45	0.43	0.73	1.04	2.14	2.51	3.47

*Surface probability is calculated using Wisconsin GCG software according to a formula of Emini et al [39].

**Antigenic index is a measure of the probability that a region is antigenic. It is calculated by summing several weighted measures of secondary structure. Wisconsin GCG software was used. The method is according to Jameson and Wolf [40].

***Hydrophilicity is calculated according to the algorithm of Hopp and Woods [41].

Table 2. Wild type and mutant truncated HPV16 L1 VLPs with and without ovalbumin were tested in ELISA for reactivity to the monoclonal antibodies H16.V5, H16.E70 and H16.D9. The results are a mean of three independent ELISAs.

Intact Constructs HPV16 $L1_{(-20)}$	Mab H16.V5*(OD_{450}) Specific for ITSE	Mab H16.E70 (OD_{450})	Mab H16.D9**(OD_{450}) Specific for disrupted VLPs
Intact V5-depleted VLPs ova$^{(-)}$	0.107	2.931	0.629
Intact V5-depleted VLPs ova$^{(+)}$	0.100	nt	0.519
Intact wild type VLPs ova$^{(-)}$	1.747	2.788	0.393
Intact wild type VLPs ova$^{(+)}$	0.661	nt	0.329
Intact HPV16 control VLPs	2.515	3.121	0.876

Disrupted Constructs HPV16 $L1_{(-20)}$	Mab H16.V5*(OD_{450}) Specific for ITSE	Mab H16.D9**(OD_{450}) Specific for disrupted VLPs
Disrupted V5-depleted VLPs ova$^{(-)}$	0.144	1.748
Disrupted V5-depleted VLPs ova$^{(+)}$	0.127	1.772
Disrupted wild type VLPs ova$^{(-)}$	0.124	2.301
Disrupted wild type VLPs ova$^{(+)}$	0.155	1.356
Disrupted HPV16 control VLPs	0.042	1.847

*V5 antibody reacts with the immunodominant type-specific epitope (ITSE) exposed on linearly assembled VLPs only. Upon concentration with VLPs the vast majority of neutralizing antibodies generated are directed against this epitope.

**D9 antibody reacts with an epitope exposed on disrupted VLPs but not on intact VLPs

Table 3a. HPV16 positive- and negative serum samples were selected from previous serological studies and tested on full length HPV16 VLPs and truncated wild type- and ITSE-depleted HPV16 VLPs. Cut-off was calculated through unit-OD approximation to 0.178.

VLPs	Serum samples from unexposed women N = 11		Serum samples from exposed women N = 49	
	Direct ELISA OD (1:30)		Direct ELISA OD (1:30)	
	mean	% positive	mean	% positive
Wild type HPV16	0.033	0	0.359	71.4
Wild type$_{(-20)}$ HPV16	0.063	9	0.307	67.3
VS-depleted$_{(-20)}$ HPV16	0.018	0	0.240	48.9

Table 3b. Neutralization serum titres based on previous results from Wang *et al* compared to percentage positive results from exposed women [33].

VLPs	Serum samples from exposed women N = 49					
	<50 neutral.titer**		50-100 neutral.titer**		≥200 neutral.titer**	
	mean OD	% positive	mean OD	% positive	mean OD	% positive
Wild type HPV16	0.237	47.3	0.322	85.7	0.536	87.5
Wild type$_{(-20)}$ HPV16	0.218	42.1	0.292	71.4	0.428	81.3
VS-depleted$_{(-20)}$ HPV16	0.139	47.3	0.250	42.9	0.324	62.5

** Neutralization titer results from Wang K, Yuh-Ying I, Pang, Schiller J.T, Lehtinen M, Dillner J, Neutralization of Papillomavirus type 16 in relation to antibody levels or avidity [33].

8. Captions to Illustrations

Figure 1: Vaccine, Janka Hlustik, Leif Dahlberg, Marie Wallen-Öhman, Joakim Dillner.
1A shows the HPV16 L1$_{(20-474)}$ monomer. The FG-loop is coloured in orange, the HI-loop is coloured in green, the DE-loop is coloured in magenta, the EF-loop is coloured in red and the BC-loop is coloured in yellow. Amino acids (aa) selected to be mutated are space filled and coloured. Aa56 is coloured yellow, aa138 is coloured magenta, aa270 is coloured blue, aa285 is coloured orange, aa348-353 are coloured green.
1B shows the side view of the HPV16 L1 pentamer. Amino acids selected to be mutated are space filled and coloured according to description in figure 1A. 1C shows the top view of the HPV16 L1 pentamer. Graphic from Rasmol.

Figure.2: Vaccine, Janka Hlustik, Leif Dahlberg, Marie Wallen-Öhman, Joakim Dillner. Detection of L1 proteins by western blotting. L1 proteins were obtained by disruption of VLPs. Lane 1) HPV16 control L1 VLPs, lane 2) truncated HPV16 L1 wild type, lane 3) truncated HPV16 L1 V5-depleted wild type, lane 4) truncated HPV16 L1 with ovalbumine, lane 5) truncated HPV16 L1 V5-depleted with ovalbumine.

Figure. 3. Vaccine, Janka Hlustik, Leif Dahlberg, Marie Wallen-Öhman, Joakim Dillner. Antibody responses induced in C57BL/6 mice. Anti-VLP reactivity was investigated by ELISA using truncated HPV16 L1 VLPs as antigen. Control mice were injected with PBS only (background). ELISA values represent the means (+/- standard deviation) of OD-values for individual mice within each immunisation group. Each immunisation group consisted of 5 mice. Truncated V5-depleteted HPV16 L1 VLPs are considerably less immunogenic than truncated wild type HPV16 L1 VLPs.

Figure 4. Vaccine, Janka Hlustik, Leif Dahlberg, Marie Wallen-Öhman, Joakim Dillner.

The monoclonal antibody .V5 was used in ELISA and heparin-based ELISA on truncated HPV16 L1 VLPs and V5-epitope depleted HPV16 L1 VLPs. HPV16 L1 VLPs lacking the last 20 amino acids at the C-terminal has a weaker binding heparin than full length HPV16 L1 VLPs. Disrupted particles were used as controls (data not shown). Bound VLPs were visualized with HPV16 specific antibodies and horseradish peroxidase coupled secondary antibody using TMB.

Figure 5. Vaccine, Janka, Leif Dahlberg, Marie Wallen-Öhman, Joakim Dillner. Illustrates specific lysis of MHC I expressing EL-4 cells by splenocytes from mice vaccinated with VLPs subcutaneously. Specific lysis was calculated using the formula [(experimental release – spontaneous release)/(maximum release – spontaneous release)] x100. As controls, lymphocytes from mice immunised with PBS and lymphocytes from mice immunised with the ovalbumin CTL-epitope/Th-peptide were analysed in parallel with those from VLP immunised mice.

9. Illustrations

Figure 1A

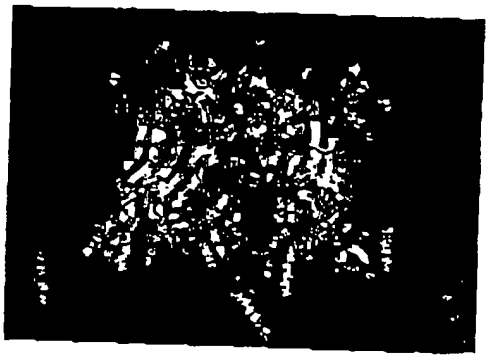

Figure 1B

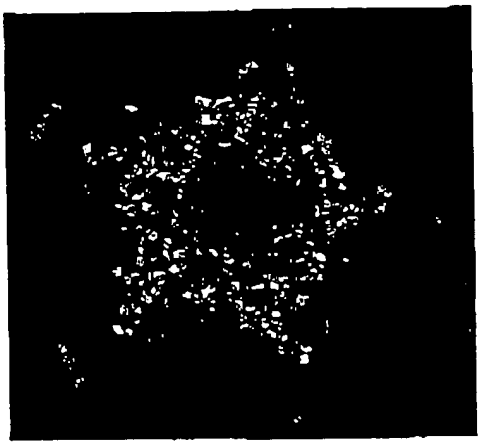

Figure 1C

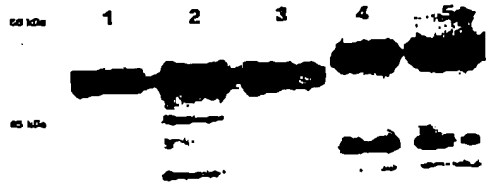

Figure 2

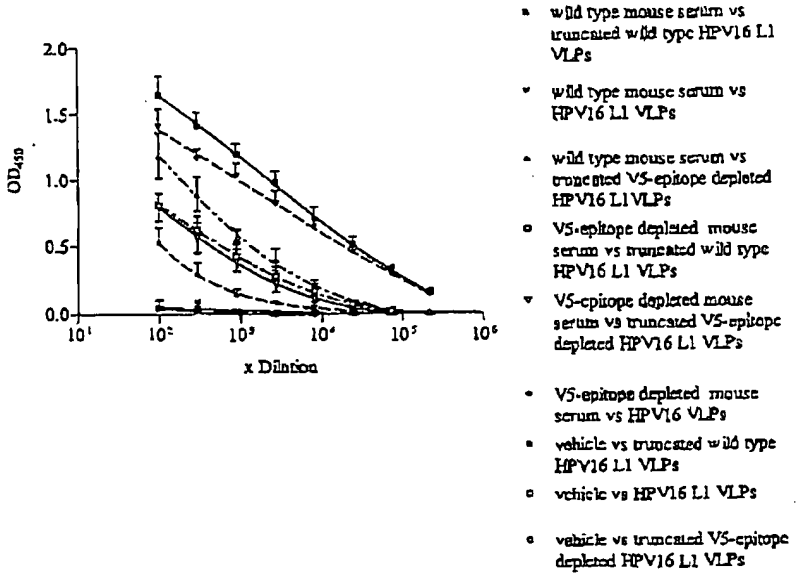
2.0
1.5
1.0
0.5
0.0
OD450
10¹
10²
10³
10⁴
10⁵
10⁶
x Dilution
wild type mouse serum vs truncated wild type HPV16 L1 VLPs
wild type mouse serum vs HPV16 L1 VLPs
wild type mouse serum vs truncated V5-epitope depleted HPV16 L1 VLPs
V5-epitope depleted mouse serum vs truncated wild type HPV16 L1 VLPs
V5-epitope depleted mouse serum vs truncated V5-epitope depleted HPV16 L1 VLPs
V5-epitope depleted mouse serum vs HPV16 VLPs
vehicle vs truncated wild type HPV16 L1 VLPs
vehicle vs HPV16 L1 VLPs
vehicle vs truncated V5-epitope depleted HPV16 L1 VLPs

Figure 3

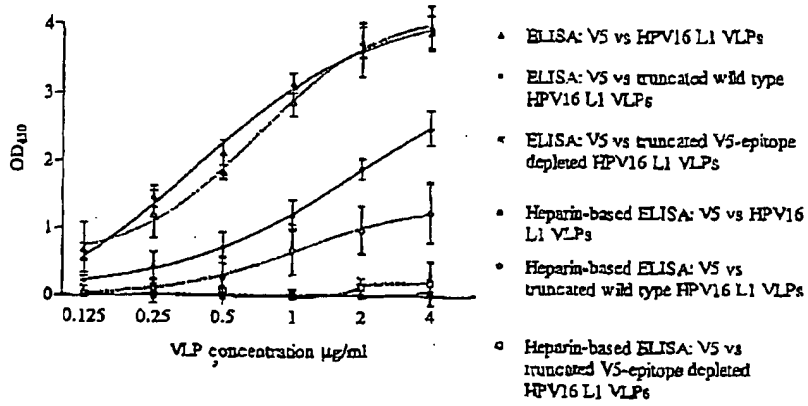
4
3
2
1
0
OD_{410}
0.125
0.25
0.5
1
2
4
VLP concentration µg/ml
ELISA: V5 vs HPV16 L1 VLPs
ELISA: V5 vs truncated wild type HPV16 L1 VLPs
ELISA: V5 vs truncated V5-epitope depleted HPV16 L1 VLPs
Heparin-based ELISA: V5 vs HPV16 L1 VLPs
Heparin-based ELISA: V5 vs truncated wild type HPV16 L1 VLPs
Heparin-based ELISA: V5 vs truncated V5-epitope depleted HPV16 L1 VLPs

Figure 4

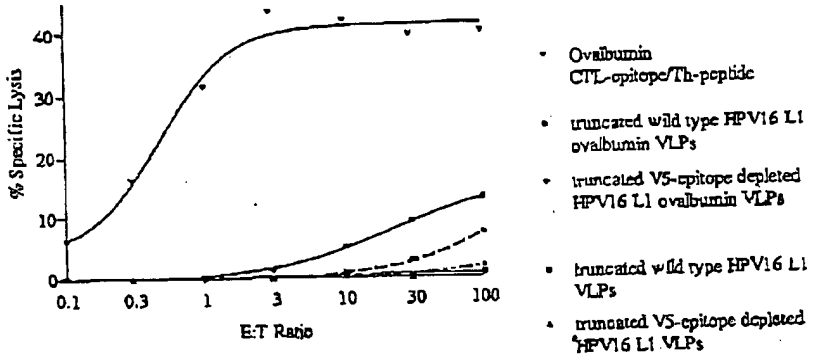
% Specific Lysis
40
30
20
10
0
0.1
0.3
1
3
10
30
100
E:T Ratio
Ovalbumin CTL-epitope/Th-peptide
truncated wild type HPV16 L1 ovalbumin VLPs
truncated V5-epitope depleted HPV16 L1 ovalbumin VLPs
truncated wild type HPV16 L1 VLPs
truncated V5-epitope depleted HPV16 L1 VLPs

Figure 5